JOURNAL OF BACTERIOLOGY, Jan. 2011, p. 575–579 Vol. 193, No. 2
0021-9193/11/$12.00 doi:10.1128/JB.01193-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

NOTES

DNA-Binding Properties of the *Bacillus subtilis* and *Aeribacillus pallidus* AC6 σ^D Proteins[▽]

Elif Sevim,[1,2,3] Ahmed Gaballa,[1]# A. Osman Beldüz,[2] and John D. Helmann[1]*

Department of Microbiology, Cornell University, Ithaca, New York[1]; Department of Biology, Karadeniz Technical University, Faculty of Arts and Sciences, Trabzon, Turkey[2]; and Department of Biology, Rize University, Faculty of Arts and Sciences, Rize, Turkey[3]

Received 5 October 2010/Accepted 8 November 2010

σ^D proteins from *Aeribacillus pallidus* AC6 and *Bacillus subtilis* bound specifically, albeit weakly, to promoter DNA even in the absence of core RNA polymerase. Binding required a conserved CG motif within the −10 element, and this motif is known to be recognized by σ region 2.4 and critical for promoter activity.

In the course of efforts to define gene expression determinants from the thermophilic bacterium *Aeribacillus pallidus* AC6 (2, 18), we identified flagellin (Hag) as being among the most highly expressed proteins in this strain. To determine the basis for high-level Hag expression, we isolated and sequenced the *hag* gene and identified and expressed the protein required for its expression in this organism, σ^D ($\sigma^D{}_{Ap}$). We here describe a comparison of $\sigma^D{}_{Ap}$ with its ortholog from *B. subtilis* ($\sigma^D{}_{Bs}$).

Cloning and sequencing of the *hag* and *sigD* genes of *A. pallidus* AC6. SDS-PAGE analysis of whole-cell lysates from *A. pallidus* AC6 identified an abundant ~35-kDa protein. The excised protein was sent to the Center of Advanced Proteomics Research Laboratory (University of Medicine and Dentistry of New Jersey) for tryptic digestion and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analyses. The resulting peptide sequences displayed high similarity to *B. subtilis* flagellin (NAQDGISLIQTAEGALTETHAILQR had 96% identity with amino acids [aa] 65 to 89 and LEHTINNL GTSAENLTAAESR had 85% identity with aa 242 to 262). Two degenerate primers (FlaF1 and FlaR1) were used to amplify the flagellin gene (*hag*) from *A. pallidus* AC6 chromosomal DNA. An ~450-bp product was cloned into pGEM-T Easy (Promega) for DNA sequencing. The remainder of *hag* and its upstream region were obtained by inverse PCR. The 828-bp *hag* gene encodes a 275-amino-acid (29.7-kDa) protein having 63% identity with *B. subtilis* Hag and is preceded by a typical σ^D promoter.

To identify *sigD*, two degenerate primers were used to amplify a PCR fragment which was cloned into the pGEM-T Easy vector system and sequenced. The flanking portions of *sigD* were obtained by inverse PCR, and the gene was sequenced. The 771-bp *sigD* gene encodes a 256-amino-acid (28.7-kDa) protein having 67% overall identity with $\sigma^D{}_{Bs}$, with the highest levels of similarity concentrated in conserved regions 2 and 4, known to mediate promoter recognition.

***A. pallidus* AC6 flagellin is expressed from a σ^D-dependent promoter.** Transcription of *hag* initiates from a canonical σ^D-dependent promoter at a G residue 79 bp upstream of the start codon (Fig. 1). Analysis of *hag*::*lacZ* fusions integrated into *B. subtilis* CU1065 and HB4035 (*sigD*::*kan*) indicated that activity was σ^D dependent and highest at late logarithmic phase, as previously reported for *B. subtilis* (19). Optimal promoter activity required an AT-rich region just upstream of the −35 element (Table 1), which has similarity with the upstream promoter (UP) element previously described for *B. subtilis hag* (6). Sequence inspection suggests that high-level Hag expression may also benefit from a strong ribosome-binding site and stabilization of the mRNA by a 5′ hairpin sequence (24).

Purification σ^D of *A. pallidus* and *B. subtilis* and reconstitution of σ^D RNAP. σ^D proteins from *A. pallidus* AC6 and *B. subtilis* were expressed under T7 RNA polymerase (RNAP) control in *Escherichia coli* BL21(DE3)/pLysS (Novagen) by using pECG1 and pECB1 (Table 2). For purification, inclusion bodies were solubilized with Sarkosyl (1), refolded, and purified using DEAE-Sepharose and heparin-Sepharose chromatography as described previously (9). *B. subtilis* core RNAP was purified from a *sigD*-null mutant expressing a His_6-tagged β′ subunit (strain EC3) by using Ni-nitrilotriacetic acid (NTA) chromatography (USB) and heparin-Sepharose chromatography (Pharmacia fast protein liquid chromatography [FPLC] system) and used to reconstitute σ^D holoenzymes (9). Both the $\sigma^D{}_{Ap}$ and $\sigma^D{}_{Bs}$ holoenzymes accurately and efficiently recognized the *A. pallidus hag* promoter on plasmid pEC3 as judged by both start site mapping (5′ rapid amplification of cDNA ends [5′-RACE]) and quantitative reverse transcription-PCR (qRT-PCR) (data not shown).

Binding affinity of σ^D of RNAP for the *hag* promoter. We have previously shown that $\sigma^D{}_{Bs}$ recognizes promoter DNA *in*

* Corresponding author. Mailing address: Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853-8101. Phone: (607) 255-6570. Fax: (607) 255-3904. E-mail: jdh9@cornell.edu.

Permanent address: Environmental Biotechnology Department, GEBRI, Mubarak City for Scientific Research and Technology Application, Borg El Arab, P.O. Box 21934, Alexandria, Egypt.

▽ Published ahead of print on 19 November 2010.

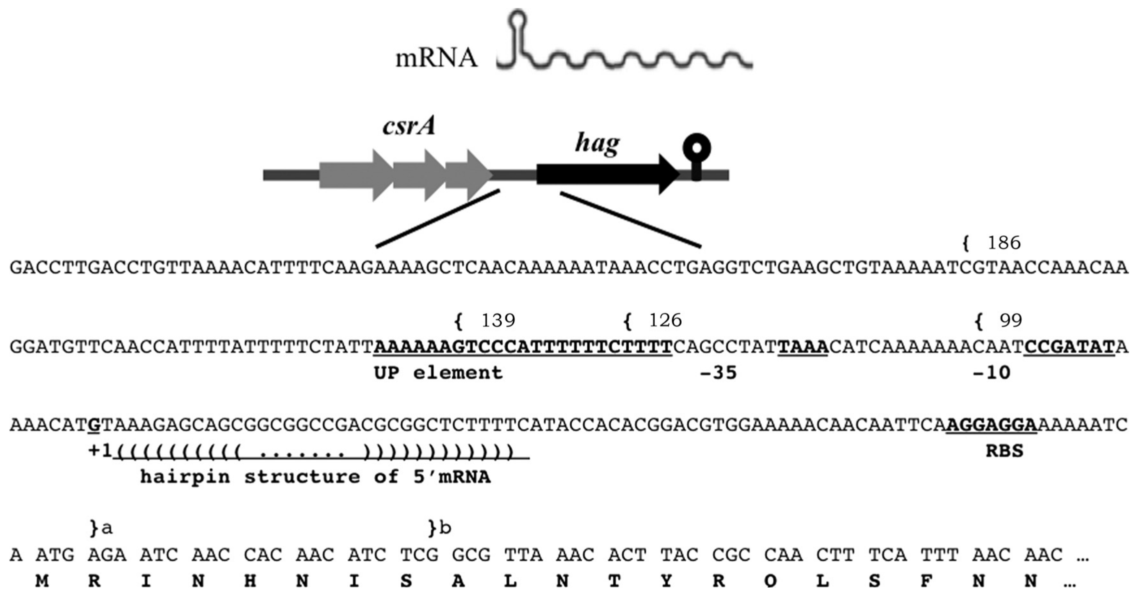

FIG. 1. The *A. pallidus hag* regulatory region. The *hag* gene is predicted to be transcribed as a monocistronic mRNA with a 5′ stem-loop (top). The regulatory region includes a predicted UP element and recognition signals (−35 and −10) for σ^D RNAP. The start site in *A. pallidus* AC6 was determined by 5′-RACE from RNA isolated from cells grown in LB medium at 60°C with shaking and corresponds to the indicated G (+1). The ribosome-binding site (RBS) and initial coding sequence are indicated. For expression studies (Table 1), promoter-*lacZ* fusions were generated from the indicated upstream endpoints ({) and either of the two downstream endpoints, designated a and b (}).

vitro in the absence of core RNAP, as judged by electrophoretic mobility shift assay (EMSA) and chemical footprinting (4). We here compared the DNA-binding abilities of $\sigma^D{}_{Ap}$ and $\sigma^D{}_{Bs}$ by using a fluorescence anisotropy-based assay with specific duplex oligonucleotides corresponding to the *A. pallidus* AC6 *hag* promoter and a control, nonspecific duplex (Fig. 2). Specific binding of $\sigma^D{}_{Ap}$ and $\sigma^D{}_{Bs}$ was apparent at several tested temperatures (Fig. 3 and data not shown). The observed affinities were relatively low (dissociation constant [K_d], ~10 to 20 μM) compared with those reported for truncated primary σ factors (5), but the specificities of the interactions were high. Previously, a somewhat higher affinity (K_d, ~1 μM) was estimated for $\sigma^D{}_{Bs}$ by using EMSA with a different labeled promoter fragment (4).

We tested two duplexes with changes within the −10 TCC GATAT consensus (M1 [TCCGCGCG] and M2 [TCTAAAT] [substitutions are underlined]; Fig. 2). Remarkably, the M2 mutation drastically affected binding by both σ factors, indicating that the CG bases within the −10 element are critical for recognition and binding. This is consistent with mutational studies demonstrating that the CG motif is critical for −10 element function (14, 28, 30). Even though M1 represents a more drastic change in sequence (a 4-bp substitution), this had a more modest effect on binding, particularly when tested with $\sigma^D{}_{Bs}$. However, a significant decrease in affinity for $\sigma^D{}_{Ap}$ was observed at elevated temperatures (Fig. 3).

Concluding remarks. Recognition of flagellin promoters by σ^D orthologs is conserved across distantly related species (3, 11, 27). We here demonstrate this conservation for *A. pallidus*; the *hag* promoter, like that in *B. subtilis* (6), is σ^D dependent and appears to include a strong UP element.

As a class, isolated σ factors are often considered to have little if any affinity for promoter DNA despite the fact that recognition of both the −35 and −10 elements involves specific σ-DNA contacts (7). DNA binding by primary σ factors (e.g., σ^{70}) can be revealed by removal of an amino-terminal domain (region 1) thought to allosterically mask the DNA-binding determinants (5). However, many alternative σ factors lack region 1, and a different mechanism of self-inhibition likely pertains. Indeed, solution studies suggest a predominant σ conformation incompatible with DNA binding (22, 25). For σ^D, structural analysis of a σ^D::FlgM complex revealed a compact σ conformation with the two DNA-binding domains (regions 2 and 4) closely apposed (26). Disulfide cross-linking suggests that a similarly compact conformation predominates in solution (25). Conversely, other studies support the idea that σ factors may specifically recognize elements of the promoter even in the absence of core RNA polymerase (12, 16, 23). We suggest that there is an equilibrium between the compact con-

TABLE 1. β-Galactosidase activity in *B. subtilis* CU1065 containing various *hag*::*lacZ* promoter fusions integrated into the *thrC* locus[a]

Promoter construct	Extent of promoter DNA (positions)	*B. subtilis* CU1065	
		β-Galactosidase activity (Miller units)	% activity
hag185	−186 to +22	1,016 ± 49	100
hag138a	−139a to +22	914 ± 99	89.9
hag138b	−139b to +4	909 ± 63	89.4
hag117a	−126a to +22	65 ± 10	6.4
hag117b	−126b to +4	63 ± 15	6.2
hag98	−99 to +22	0	0
hag56	−57 to +22	0	0

[a] See also Table 2. Strains were grown in Schaeffer's sporulation medium (21), samples were collected in late logarithmic phase, and activity was measured using the method of Miller (17). Values reported are averages and standard deviations of triplicate determinations.

TABLE 2. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or relevant characteristics[a]	Reference, source, or purpose
Bacillus subtilis		
CU1065	W168 *trpC2 attSP*β	Lab stock
HB4035	CU1065 *sigD*::*kan*	Lab stock
HB7707	JH642 *trpC2 pheA1 rpoC*::His_6 Spc^r	Lab stock
EC3	HB4035 *rpoC*::His_6 Spt^r Kan^r	This study
Plasmids		
pDG1663	Integrational plasmid (inserts at *thrC* locus)	8
pECG1	pET11a carrying *sigD* gene of *A. pallidus* AC6	This study
pECB1	pET11a carrying *sigD* gene of *B. subtilis* CU1065	This study
pEC3	pDG1663 containing *hag* promoter from −185 to +385 from start codon	This study
pEC3-185 series	Series of pDG1663 derivatives with truncated *hag* promoters as EcoRI-HindIII fragments (suffix indicating the upstream endpoint)	
Primers		
FlaF1	GCNGGNGAYGAYGCNGCNGGNYTNGC	*hag* (degenerate)
FlaR1	GTNCCNARRTTRTTDATNGTRTGYTC	*hag* (degenerate)
SigDF2	AARTTYGAYACNTAYGCNTCNTTYMG	*sigD* (degenerate)
SigDR2	GMRTGDATYTGNGADATNCKNGANGTNG	*sigD* (degenerate)
Gpa sigDf	TATCAC*CATATG*ATGGTCCAATCGATGACACTG	Cloning into pET11a
Gpa sigDr	TAT*GGATCC*TTAAGATAAAAGCTTAACGAGC	Cloning into pET11a
Bsu sigDf	TATCAC*CATATG*ATGCAATCCTTGAATTATGAAG	Cloning into pET11a
Bsu sigDr	TAT*GGATCC*TTATTGTATCACTTTTTCCAGC	Cloning into pET11a

[a] Italics indicates a restriction site. Spt^r, spectinomycin resistant; Kan^r, kanamycin resistant.

formation, unable to interact specifically with DNA, and a more open conformation in which specific DNA binding is possible.

The −10 element is highly conserved in σ^D promoters. The GCCG motif is a composite recognition element: the GC is recognized by R91 from region 3 of *E. coli* σ^{28} (the σ^D ortholog), whereas the CG is recognized by R84 and D81 from region 2.4 (14). The corresponding residues in σ^D_{Ap} are R104

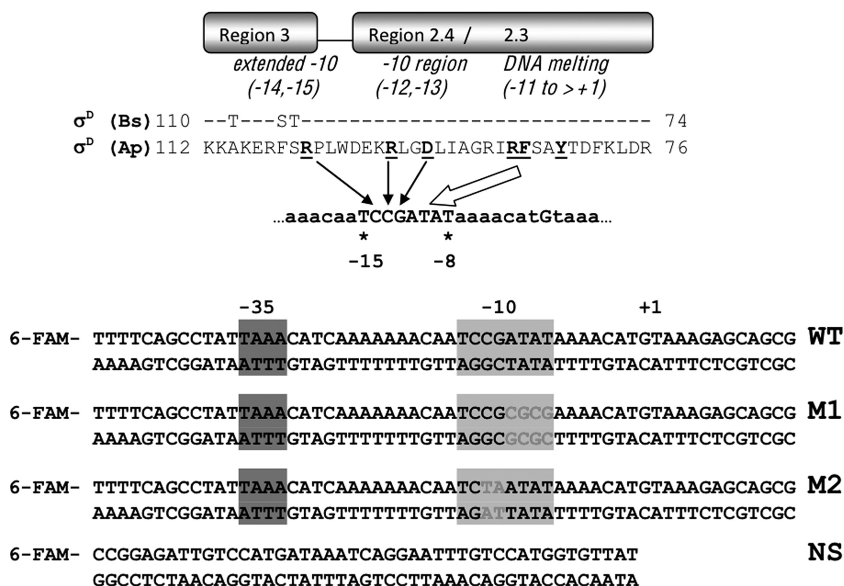

FIG. 2. Promoter recognition by σ^D proteins. The −35 region is recognized by region 4.3 (not shown), and the −10 element is recognized by region 2 and an Arg residue from the amino terminus of region 3 (14). The σ^D_{Ap} and σ^D_{Bs} proteins are aligned from the initial portion of region 3 through region 2.3 (note that the direction of the protein sequences is inverted relative to conventional orientation). There are only three amino acid substitutions in this region (identical residues are indicated by dashes) and all residues known to contact DNA are identical. The underlined residues implicated in sequence specific promoter recognition include (σ^D_{Ap} numbering) R104 in region 3 and R97 and D94 in region 2.4. Residues in region 2.3 corresponding to positions involved in promoter melting in other σ factors are also underlined (15). Fluorescently labeled (6-carboxyfluorescein [6-FAM]) oligonucleotide duplexes were used for fluorescence anisotropy analysis of σ-DNA interactions. The −35 and −10 elements are shaded, and the substituted bases are highlighted. +1 indicates the start site for transcription. WT indicates the wild type. M1 and M2 are mutants with mutations in the core −10 element, and NS represents a nonspecific control DNA.

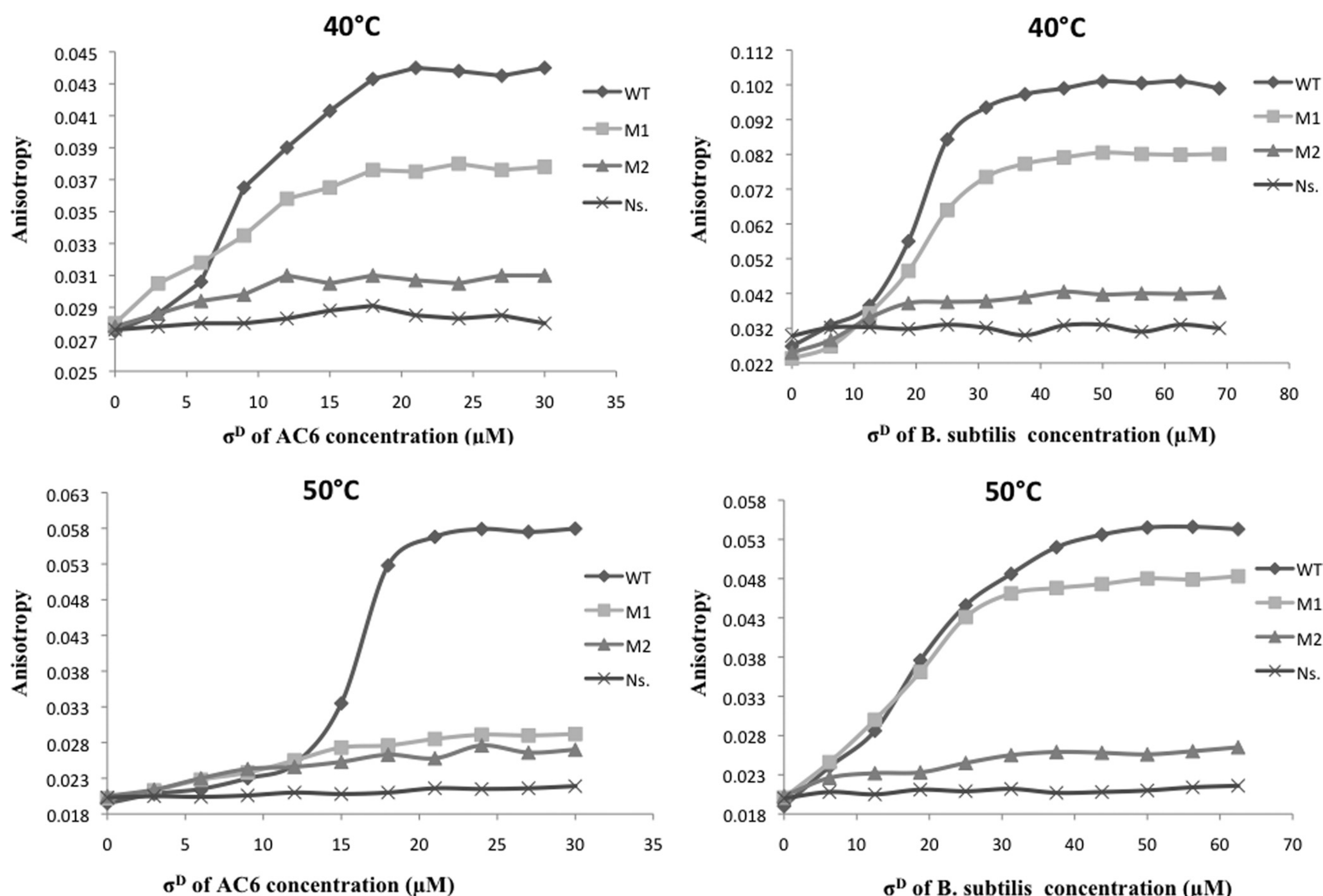

FIG. 3. DNA binding by purified σ^D proteins monitored by fluorescence anisotropy (FA). σ^D proteins were added stepwise from 0 to 60 μM, and DNA binding was detected as an increase in anisotropy of the probe DNA. Probe DNA was a 50-bp 6-carboxyfluorescein (6-FAM)-labeled *hag* duplex oligonucleotide (Fig. 2) or a variant altered in the −10 region (M1 [from TCCGATAT to TCCGCGCG] and M2 [from TCCGATAT to TCTAATAT] [substitutions are underlined]). FA experiments (excitation wavelength [λ_{ex}], 492 nm [slit width = 10 nm]; emission wavelength [λ_{em}], 492 nm [slit width = 15 nm]) were performed at 40 or 50°C in 100 μl TGED buffer (10 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol [DTT]) with 100 nM DNA and 50 mM NaCl. Averages of 6 measurements with an integration time of 9 s were determined.

from region 3 and R97 and D94 from region 2.4. *E. coli* σ^{28} R91 recognizes a G residue at either of the first two positions of the extended −10 element (15), and σ^D_{Ap} R104 is therefore predicted to contact G on the template strand at position −14 (Fig. 2). The neighboring AT-rich motif (ATAT) likely functions in DNA melting, presumably via interaction with region 2.3 (13, 15). In general, it is not yet known whether the downstream portion of the −10 element is recognized as duplex DNA or whether this region establishes close interactions with σ only after promoter melting. For nearly all σ factors, this region is AT rich (and often includes alternating AT residues). Since it is unlikely that σ factor alone can melt DNA (29), this may explain the relatively modest effects of the substitutions in M1 on binding by σ^D_{Bs}. Conversely, the notable effect of the M1 substitution on DNA binding by σ^D_{Ap} at elevated temperatures hints that duplex recognition of this region may also play a role.

In sum, these results suggest that σ^D proteins may prove to be a useful model system for investigation of σ-DNA recognition. Despite recent progress in RNAP structural biology (reviewed in reference 20), we still lack a high-resolution view of key transcription intermediates in open-complex formation. Development of simplified model systems is one promising approach for dissecting the interactions that occur during transcription initiation (10, 23, 29).

Nucleotide sequence accession numbers. The *hag* and *sigD* gene sequences have been submitted to GenBank under accession numbers GU991850 and HM126480, respectively.

This work was supported by a fellowship from the Scientific and Technological Research Council of Turkey (Tubitak) to E.S. and a grant from the NIH (GM047446) to J.D.H.

REFERENCES

1. **Burgess, R. R.** 1996. Purification of overproduced *Escherichia coli* RNA polymerase sigma factors by solubilizing inclusion bodies and refolding from Sarkosyl. Methods Enzymol. **273:**145–149.
2. **Canakci, S., K. Inan, M. Kacagan, and A. O. Belduz.** 2007. Evaluation of arabinofuranosidase and xylanase activities of *Geobacillus* spp. isolated from some hot springs in Turkey. J. Microbiol. Biotechnol. **17:**1262–1270.
3. **Chen, Y. F., and J. D. Helmann.** 1992. Restoration of motility to an *Escherichia coli fliA* flagellar mutant by a *Bacillus subtilis* sigma factor. Proc. Natl. Acad. Sci. U. S. A. **89:**5123–5127.
4. **Chen, Y. F., and J. D. Helmann.** 1995. The *Bacillus subtilis* flagellar regulatory protein σ^D: overproduction, domain analysis and DNA-binding properties. J. Mol. Biol. **249:**743–753.

5. **Dombroski, A. J., W. A. Walter, M. T. Record, Jr., D. A. Siegele, and C. A. Gross.** 1992. Polypeptides containing highly conserved regions of transcription initiation factor sigma 70 exhibit specificity of binding to promoter DNA. Cell **70:**501–512.
6. **Fredrick, K., T. Caramori, Y. F. Chen, A. Galizzi, and J. D. Helmann.** 1995. Promoter architecture in the flagellar regulon of *Bacillus subtilis*: high-level expression of flagellin by the sigma D RNA polymerase requires an upstream promoter element. Proc. Natl. Acad. Sci. U. S. A. **92:**2582–2586.
7. **Gruber, T. M., and C. A. Gross.** 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. **57:**441–466.
8. **Guérout-Fleury, A. M., N. Frandsen, and P. Stragier.** 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57–61.
9. **Helmann, J. D.** 2003. Purification of *Bacillus subtilis* RNA polymerase and associated factors. Methods Enzymol. **370:**10–24.
10. **Helmann, J. D., and P. L. deHaseth.** 1999. Protein-nucleic acid interactions during open complex formation investigated by systematic alteration of the protein and DNA binding partners. Biochemistry **38:**5959–5967.
11. **Heuner, K., J. Hacker, and B. C. Brand.** 1997. The alternative sigma factor σ^{28} of *Legionella pneumophila* restores flagellation and motility to an *Escherichia coli fliA* mutant. J. Bacteriol. **179:**17–23.
12. **Imashimizu, M., M. Hanaoka, A. Seki, K. S. Murakami, and K. Tanaka.** 2006. The cyanobacterial principal sigma factor region 1.1 is involved in DNA-binding in the free form and in transcription activity as holoenzyme. FEBS Lett. **580:**3439–3444.
13. **Juang, Y. L., and J. D. Helmann.** 1994. A promoter melting region in the primary sigma factor of *Bacillus subtilis*. Identification of functionally important aromatic amino acids. J. Mol. Biol. **235:**1470–1488.
14. **Koo, B. M., V. A. Rhodius, E. A. Campbell, and C. A. Gross.** 2009. Mutational analysis of *Escherichia coli* σ^{28} and its target promoters 'reveals recognition of a composite −10 region, comprised of an 'extended −10' motif and a core −10 element. Mol. Microbiol. **72:**830–843.
15. **Koo, B. M., V. A. Rhodius, G. Nonaka, P. L. deHaseth, and C. A. Gross.** 2009. Reduced capacity of alternative sigmas to melt promoters ensures stringent promoter recognition. Genes Dev. **23:**2426–2436.
16. **Kudo, T., D. Jaffe, and R. H. Doi.** 1981. Free sigma subunit of *Bacillus subtilis* RNA polymerase binds to DNA. Mol. Gen. Genet. **181:**63–68.
17. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. **Minana-Galbis, D., D. L. Pinzon, J. G. Loren, A. Manresa, and R. M. Oliart-Ros.** Reclassification of *Geobacillus pallidus* (Scholz et al. 1988) Banat et al. 2004 as *Aeribacillus pallidus* gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. **60:**1600–1604.
19. **Mirel, D. B., et al.** 2000. Environmental regulation of *Bacillus subtilis* σ^{D}-dependent gene expression. J. Bacteriol. **182:**3055–3062.
20. **Murakami, K. S., and S. A. Darst.** 2003. Bacterial RNA polymerases: the wholo story. Curr. Opin. Struct. Biol. **13:**31–39.
21. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. U. S. A. **54:**704–711.
22. **Schwartz, E. C., et al.** 2008. A full-length group 1 bacterial sigma factor adopts a compact structure incompatible with DNA binding. Chem. Biol. **15:**1091–1103.
23. **Sevostyanova, A., et al.** 2007. Specific recognition of the −10 promoter element by the free RNA polymerase sigma subunit. J. Biol. Chem. **282:**22033–22039.
24. **Sharp, J. S., and D. H. Bechhofer.** 2005. Effect of 5'-proximal elements on decay of a model mRNA in *Bacillus subtilis*. Mol. Microbiol. **57:**484–495.
25. **Sorenson, M. K., and S. A. Darst.** 2006. Disulfide cross-linking indicates that FlgM-bound and free σ^{28} adopt similar conformations. Proc. Natl. Acad. Sci. U. S. A. **103:**16722–16727.
26. **Sorenson, M. K., S. S. Ray, and S. A. Darst.** 2004. Crystal structure of the flagellar sigma/anti-sigma complex σ^{28}/FlgM reveals an intact sigma factor in an inactive conformation. Mol. Cell **14:**127–138.
27. **Studholme, D. J., and M. Buck.** 2000. The alternative sigma factor σ^{28} of the extreme thermophile *Aquifex aeolicus* restores motility to an *Escherichia coli fliA* mutant. FEMS Microbiol. Lett. **191:**103–107.
28. **Wozniak, C. E., and K. T. Hughes.** 2008. Genetic dissection of the consensus sequence for the class 2 and class 3 flagellar promoters. J. Mol. Biol. **379:**936–952.
29. **Young, B. A., T. M. Gruber, and C. A. Gross.** 2004. Minimal machinery of RNA polymerase holoenzyme sufficient for promoter melting. Science **303:**1382–1384.
30. **Yu, H. H., E. G. Di Russo, M. A. Rounds, and M. Tan.** 2006. Mutational analysis of the promoter recognized by *Chlamydia* and *Escherichia coli* σ^{28} RNA polymerase. J. Bacteriol. **188:**5524–5531.